HLA-DRB

BigDye™ Terminator Sequencing-Based Typing Kit

Protocol

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Applied Biosystems

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Introduction

Overview

IMPORTANT This kit should be used in accordance with guidelines established by the Committee for Quality Assurance and Standards of the American Society for Histocompatibility and Immunogenetics.[1]

The genetic complexity of the DRB region of the human major histocompatibility complex (MHC) has required development of molecular typing techniques with increasing levels of resolution. Methods such as Polymerase Chain Reaction–Sequence-Specific Oligonucleotide Probe (PCR-SSOP) or Sequence-Specific Primer–PCR (SSP-PCR) are not always able to discriminate among the approximately 200 recognized alleles at DRB1.

To address this need, Applied Biosystems has developed a system that combines a low-resolution SSP-PCR followed by high-resolution allele typing using automated DNA sequencing.

SSP-PCR Amplifications

The low resolution SSP-PCRs are based on allele group-specific motifs in the first hypervariable region of exon 2. The following allele groups and genes are amplified with the eleven specific PCR mixes provided:

- DR1
- DR2
- DR3/11/6
- DR4
- DR7
- DR8/12
- DR9
- DR10
- DRB3
- DRB4
- DRB5

In addition to the group-specific amplification mixes, a control mix is also provided that amplifies all DRB alleles and genes (DRB*ALL). This mix can be used as either a positive or negative amplification control. These PCRs have been optimized for use with AmpliTaq Gold® DNA Polymerase to ensure specific and efficient reactions.

1. 1996 ASHI Standards for Histocompatibility Testing. *American Society for Histocompatibility and Immunogenetics, 1996 Membership Directory*, pp. 17–28.

Sequencing SSP-PCR Fragments.

The positive amplification reactions from the SSP-PCR are used as sequencing templates to generate the high resolution allele typing information. The PCR and sequencing protocols enable direct sequencing with no PCR purification steps. The design of the amplification primers allows sequencing of both strands from a single amplification fragment. The BigDye™ terminator cycle sequencing chemistry, provided in the Ready Reaction format, has been optimized for ease of use and high throughput with less technician time.

Allele Assignment

The final step in the analysis procedure is to perform the allele assignment using MatchTools™ Software and MTNavigator™ Software provided in the MatchMaker™ Allele Identification Software package. These programs work together to assign alleles and to allow manual review or editing of the sequencing data.

Instrument Platforms

The protocols provided in this document were optimized using the GeneAmp® PCR System 2400 and GeneAmp® PCR System 9600 Thermal Cyclers. Specific instructions are given for the HLA-DRB kit reagents to generate data on the ABI PRISM® 310 Genetic Analyzer, and the ABI PRISM® 377 DNA Sequencer. The protocol identifies certain steps where the protocol varies between the instruments. For more detailed instructions on using the instruments, refer to the appropriate instrument user's manual.

IMPORTANT This kit is not designed for use with the ABI™ 373 DNA Sequencer or the ABI 373 DNA Sequencer with the XL Upgrade.

Materials and Equipment

Reagents Included

The HLA-DRB BigDye™ Terminator Sequencing-Based Typing Kit includes the reagents listed below. One kit is sufficient for generating complete HLA-DRB typings for 48 purified DNA samples.

- HLA-DRB Amplification Module (for amplification of 48 purified DNAs)
  - One tube of AmpliTaq Gold DNA Polymerase (50 µL, 5 U/µL)
  - 12 tubes of PCR Mixes, one for each SSP-PCR
- HLA-DRB Sequencing Module (sufficient for sequencing 96 templates generated with the HLA-DRB Amplification Module)
  - Two tubes of HLA-DRB Forward Sequencing mix
  - Two tubes of HLA-DRB Reverse Sequencing mix
  - Two tubes of DNA Diluent Buffer

Reagents and Equipment Not Included

In addition to the reagents supplied in this kit, other items are required depending on which sequencing instrument is used. Refer to the individual instrument manuals for the specific items needed. Many of the items listed are available from major laboratory suppliers (MLS) unless otherwise noted. Equivalent sources may be acceptable where noted.

Control and Loading Buffer Module

The Control and Loading Buffer Module (P/N M0028) is sold separately and contains the following:

- Two tubes of human DNA, male (DRB1*0301/1001, DRB3*0202) (10 ng/µL, 100 µL each tube)
- Five tubes of recrystallized formamide (0.5 mL each)
- One tube of blue dextran/EDTA solution (1.0 mL)

Table 1. Reagents Supplied by the User

Reagent	Source
ABI PRISM dRhodamine Matrix Standards Kit	Applied Biosystems (P/N 403047)
Agarose gel loading buffer	MLS

Table 1. Reagents Supplied by the User *(continued)*

Reagent	Source
Agarose, molecular biology grade	MLS
Boric acid	MLS
Deionized water	MLS
Disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA•2H_2O$)	Sigma (P/N E4884) or Gibco BRL (P/N SS7SUA)
DNA size ladders, 1 µg/µL, 100-bp or 250-bp	Gibco BRL (P/N 15628-050, 10596-013)
Ethanol (absolute)	MLS
Ethanol (anhydrous)	Kodak IBI (P/N IB15720)
Ethidium bromide	Sigma (P/N E1510) or MLS
Sodium acetate, 3 M, pH 4.6	Applied Biosystems (P/N 400320)
Tris base, molecular biology grade	MLS
Tris-HCl, molecular biology grade	MLS
For the ABI PRISM 310 Genetic Analyzer Only	
10X Genetic Analyzer Buffer with EDTA	Applied Biosystems (P/N 402824)
Performance Optimized Polymer 6 (POP-6™)	Applied Biosystems (P/N 402837)
For the ABI PRISM 377 DNA Sequencer Only:	
Ammonium persulfate	Kodak IBI (P/N IB70080)
Long Ranger Gel Solution, 50%	FMC (P/N 50611)
Long Ranger Singel pack Type: 377-36 cm WTR	FMC (P/N 40691)
N,N,N´,N´-Tetramethylethylenediamine (TEMED)	Kodak IBI (P/N IB70120)
Urea	Kodak IBI (P/N IB72064)

Table 2. Equipment Supplied by the User

Item	Source
ABI PRISM 377 DNA Sequencer, or ABI PRISM 310 Genetic Analyzer	Applied Biosystems
ABI PRISM® DNA Sequencing Analysis Software, v. 2.1.1, v. 2.1.2, or v. 3.0	Applied Biosystems
GeneAmp PCR System 2400 or 9600	Applied Biosystems
Heating block	MLS
MatchMaker™ Allele Identification Software, v. 1.0 (includes MatchMaker™, MatchTools™ and MTNavigator™ software)	Applied Biosystems (P/N M0024)
MicroAmp® 2400 or 9600 Base	Applied Biosystems (P/N N801-0531, N801-5531)
MicroAmp® 2400 or 9600 Tray/Retainer Set	Applied Biosystems (P/N N801-0530, N801-5530)
MicroAmp® 2400 or 9600 Full Plate Cover	Applied Biosystems (P/N N801-0550, N801-5550)
MicroAmp® caps, 8 or 12/strip	Applied Biosystems (P/N N801-0535, N801-0534)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
MicroAmp® Reaction Tubes, 0.2-mL, or strip tubes	Applied Biosystems (P/N N801-0533, N801-0580)
Multichannel pipettor	MLS
Pipettes and tips	MLS
Spin Column, Centri-Sep™, 1-mL	Princeton Separations (P/N CS-901) Outside North America: Applied Biosystems (P/N 401763, P/N 401762)
Tabletop centrifuge, with 96-well microtiter tray adaptor	MLS
Vortexer	MLS

Table 2. Equipment Supplied by the User *(continued)*

Item	Source
For the ABI PRISM® 310 Genetic Analyzer Only	
ABI PRISM 310 Genetic Analyzer capillary, labeled with a green mark (L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm)	Applied Biosystems (P/N 402839)
ABI PRISM 310 Genetic Analyzer Sample Tubes, 0.5-mL	Applied Biosystems (P/N 401957)
ABI PRISM 310 Genetic Analyzer septa for 0.5-mL sample tubes	Applied Biosystems (P/N 401956)
ABI PRISM 310 Genetic Analyzer Buffer Vials, 4.0-mL	Applied Biosystems (P/N 401955)
Genetic Analyzer Retainer Clips	Applied Biosystems (P/N 402866)
Genetic Analyzer Septa Strips, 0.2 mL Tube	Applied Biosystems (P/N 402059)
Glass syringe, 1.0-mL	Applied Biosystems (P/N 604418)

Safety Information

For information on the safe operation of the ABI PRISM 310 Genetic Analyzer, refer to the *ABI PRISM 310 Genetic Analyzer Site Preparation and Safety Guide.*

For information on the safe operation of the ABI PRISM 377 DNA Sequencer, refer to the *ABI PRISM 377 DNA Sequencer Safety Summary.*

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For Support On This Product	Dial 1-800-831-6844, and...	
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	1-800-952-4716	650-638-6223 9:00 a.m. to 5:00 p.m. PT

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by phone from outside the United States or Canada	a. Dial your international access code, then 1-858-712-0317, from a touch-tone phone. Have your complete fax number and country code ready (011 precedes the country code). b. Press **1** to order an index of available documents and have it faxed to you. Each document in the index has an ID number. (Use this as your order number in step “d” below.) c. Call 1-858-712-0317 from a touch-tone phone a second time. d. Press **2** to order up to five documents and have them faxed to you.

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Czech Republic and Slovakia (Praha) Tel: 420 2 61 222 164 Fax: 420 2 61 222 168	The Netherlands (Nieuwerkerk a/d IJssel) Tel: 31 (0)180 331400 Fax: 31 (0)180 331409
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France (Paris) Tel: 33 (0)1 69 59 85 85 Fax: 33 (0)1 69 59 85 00	Portugal (Lisboa) Tel: 351 (0)22 605 33 14 Fax: 351 (0)22 605 33 15
Germany (Weiterstadt) Tel: 49 (0) 6150 101 0 Fax: 49 (0) 6150 101 101	Russia (Moskva) Tel: 7 095 935 8888 Fax: 7 095 564 8787
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Korea (Seoul) Tel: 82 2 593 6470/6471 Fax: 82 2 593 6472	Thailand (Bangkok) Tel: 66 2 719 6405 Fax: 66 2 319 9788

Blood Collection and DNA Sample Preparation

Procedure

! WARNING ! BIOHAZARD. Blood samples have the potential to transmit infectious diseases. Follow the latest guidelines published by the Centers for Disease Control (CDC) and National Institutes of Health (NIH) concerning the principles of risk assessment, biological containment, and safe laboratory practices for activities involving clinical specimens. These principles can be found in the U.S. Department of Health and Human Services (HHS) publication, *Biosafety in Microbiological and Biomedical Laboratories* **(publication number 93-8395, stock number 017-040-523-7). The biosafety Level-2 containment elements are consistent with the Occupational Safety and Health Administration (OSHA) requirements contained in the HHS OSHA** *Bloodborne Pathogen Standard 29 CFR*, **part 1910.1030.**

To collect and prepare blood samples :

Step	Action
1	Collect a sample of venous blood into an ACD-A, EDTA, or heparin tube.
2	Prepare genomic DNA following appropriate blood handling procedures. The following methods have been shown to yield high-quality, high-molecular-weight genomic DNA directly from samples of fresh blood: ♦ QiaAmp Blood Kit (Qiagen, P/N 29104) ♦ PureGene (Gentra, P/N D5000) ♦ Isoquick Nucleic Acid Extraction Kit (Orca, P/N MXT-020-100) ♦ Manual salting out[a,b] These DNA purification kits provide DNA that works well with the Applied Biosystems HLA typing kits. Other methods may work, but should be validated before routine use.

To collect and prepare blood samples *(continued)*:

Step	Action
3	Prepare DNA dilution buffer (10 mM of Tris-HCl, 0.1 mM of EDTA, pH 8.0).
4	Quantitate and resuspend purified DNA in DNA dilution buffer to a concentration of 10 ng/µL.
5	Store at 2–6 °C.

a. Miller, S. A., Dykes, D. D. and Polesky, H. F., 1988. “A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells.” *Nucleic Acids Research* 16: 1215.

b. *Proceedings of the Eleventh International Histocompatibility Workshop and Conference,* Yokohama, Japan, November 6–13, 1991.

Low-resolution SSP-PCR DRB Typing

Overview

The low resolution DRB typing is based on the SSP-PCR results generated with the reagents in the HLA-DRB Amplification Module. For each DNA sample to be tested, 12 separate PCR amplifiications are performed.

IMPORTANT These manipulations must be performed in an environment free of contamination by human DNA or PCR amplicons, such as a separate clean room or biological containment hood. Vortex all reagents briefly before using.

Low-resolution SSP-PCR

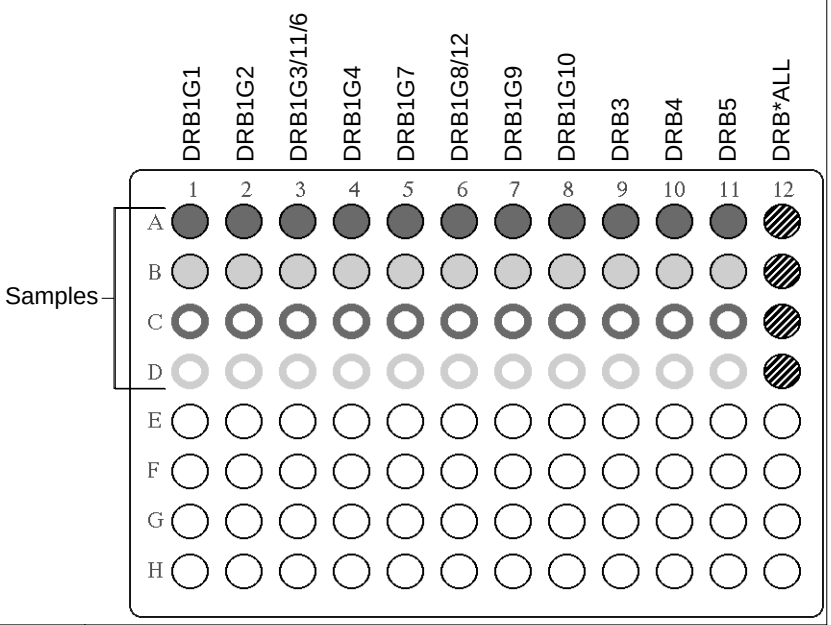
To perform low-resolution SSP-PCR

Step	Action	
1	Prepare samples at room temperature as follows:	
	IF preparing…	**THEN…**
	Allele Group-specific Reactions (DRB1G1, DRB1G2, DRB1G3/11/6, DRB1G4, DRB1G7, DRB1G8/12, DRB1G9, DRB1G10, DRB3, DRB4, and DRB5)	a. Add 1.0 µL AmpliTaq Gold DNA Polymerase (stock solution at 5 U/µL) to 40 µL genomic template (10 ng/µL). b. Add 10 µL of each Allele Group-specific PCR Mix to 0.2-mL MicroAmp® PCR tubes. c. Add 2.5 µL of genomic template/AmpliTaq Gold DNA Polymerase mixture to the PCR mixes.
	Positive Control Reactions (DRB*ALL)	Add 2.5 µL of the genomic template/AmpliTaq Gold DNA Polymerase mixture to a 0.2-mL MicroAmp PCR tube containing 10 µL of the DRB*ALL PCR mix.

To perform low-resolution SSP-PCR *(continued)*

Step	Action	
	Negative Control Reactions (DRB*ALL)	Combine 1.0 µL of AmpliTaq Gold DNA Polymerase with 40 µL DNA Dilution Buffer. Add 2.5 µL of DNA Dilution Buffer/AmpliTaq Gold DNA Polymerase mixture to a 0.2-mL MicroAmp PCR tube containing 10 µL of DRB*ALL PCR Mix.
	Note Using the DRB*ALL PCR as a positive or negative control is up to the individual user. If detecting contamination is critical, then using the DRB*ALL as a negative control is appropriate. If it is important to ensure that the DNA sample will in fact work in the amplification reactions, then it may be more appropriate to use the DRB*ALL as a positive control.	
	Note An indicator dye may be used by adding 1.2 µL of a 25 mM solution of cresol red dye to the genomic template/AmpliTaq Gold mixture. The genomic template/AmpliTaq mixture has been added to the PCR reactions if the final solutions have slight red tints.	
2	Place the tubes in a 96-well or 24-well tube tray/retainer depending on the thermal cycler to be used. A suggested tube arrangement for four DNA specimens in a 96-well tray is shown below. Each row corresponds to a different sample. The first eleven columns in each row contain Allele Group-specific PCR Mix, genomic template, and AmpliTaq Gold DNA Polymerase. The twelfth column contains DRB*ALL PCR Mix as either a positive or negative control.	

To perform low-resolution SSP-PCR *(continued)*

Step	Action
	
3	Spin tubes briefly in a tabletop centrifuge fitted with a 96-well or 24-well tray support to collect their contents at the bottoms of the tubes.
4	Seal the tubes with MicroAmp Caps or cover them with a MicroAmp 9600 or 2400 Full Plate Cover.
5	Place the tray in the thermal cycler (GeneAmp PCR System 9600, or 2400).

Thermal Cycling Conditions

For either the GeneAmp PCR System 9600 or 2400, set the reaction volume to 12 µL.

Program the thermal cycler to:

Step	Action
1	Do 1 cycle of the following to activate AmpliTaq Gold: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 10 min.
2	Repeat the following for 36 cycles: ♦ 96 °C for 20 seconds ♦ Rapid thermal ramp to 65 °C ♦ 65 °C for 30 seconds ♦ Rapid thermal ramp to 72 °C ♦ 72 °C for 30 seconds
3	Do 1 cycle of the following: Rapid thermal ramp to 99 °C 99 °C for 10 min.
4	Do 1 cycle of the following: ♦ Rapid thermal ramp to 4 °C ♦ 4 °C forever
5	After amplification, store PCR products at 2–6 °C until needed.

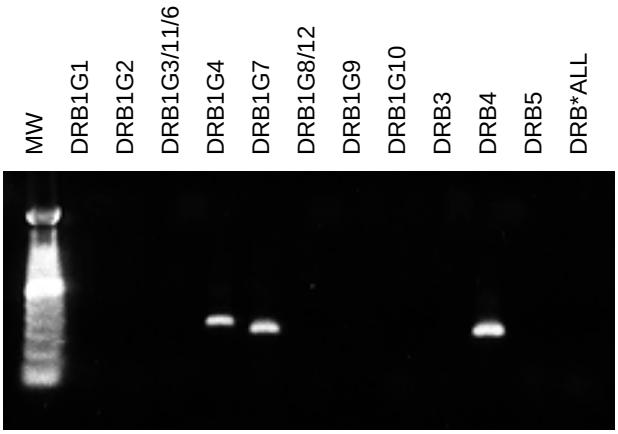
Analysis of Low-resolution PCR Amplifications

Protocol

To analyze low-resolution PCR amplifications:

Step	Action
1	Prepare a 2% agarose gel containing 1X TBE buffer and 0.5 µg/mL ethidium bromide. **! WARNING ! CHEMICAL HAZARD. Ethidium bromide is a known mutagen. It can change genetic material in a living cell. Before using ethidium bromide as a stain, read the manufacturer's MSDS, which gives information on physical characteristics, hazards, precautions, first-aid, spill clean-up, and disposal procedures. Wear appropriate protective eyewear, clothing, and gloves.**
2	Combine 7.5 µL of each PCR amplification reaction with 3 µL of agarose gel loading buffer.
3	Load 10 µL on the agarose gel.
4	Load in one lane 0.5 µg of BRL 100-bp or 250-bp size ladder in loading buffer.
5	Run the gel in 1X TBE at 10 V/cm until the fragments in the size ladder resolve. For example, a 100 mL, 10 5 cm submarine gel run at 150 V for 30 minutes is sufficient.

To analyze low-resolution PCR amplifications: *(continued)*

Step	Action
6	Photograph the resulting electrophoresis pattern. The photograph below shows that for this sample, the DRB1G4, DRB1G7, and DRB4 alleles are present. 
7	Record the electrophoresis results on a form like the one in Appendix A on page 44.
8	Compare the results with the table in Appendix B on page 45. **Note** An amplification reaction is considered positive if an SSP-PCR fragment has an intensity comparable to either the 600-bp fragment of the 100-bp ladder, or the 1000-bp fragment of the 250-bp ladder.

Low-resolution Results as Control Reactions

Considerations to keep in mind while reviewing the low resolution results include the following:

- Only one or two of the DRB1 PCRs should be positive. If three or more DRB1 amplifications are positive, then contamination or a PCR problem may exist. If none is positive, then there may be a problem with the sample, the reagents, and/or the thermal cycler.
- The positive DRB1 PCRs should be consistent with the DRB3, DRB4, and DRB5 results based on known linkage patterns. These patterns are detailed in the table in Appendix B on page 45. For example, refer to the figure in step 6 on page 21. Note that DRB1G4 and DRB1G7 are positive and that DRB4 is also positive.
- Known exceptions to these rules include the few alleles that do not amplify in the expected allele groups as listed below.

DRB1G8/12	DRB1G4
1105	1122
1317	1410
1404	
1415	
1428	
1431	

- Other DRB haplotypes may have an unusual amplification pattern. If a combination is observed that is not consistent with the table on page 45, then:
  - Record your observations.
  - Check your results carefully after sequencing and allele assignment.
  - Consult the latest allele sequence layouts and scientific literature.

    For example, some DR2 alleles are on haplotypes that lack the DRB5 gene.

Forward and Reverse Sequencing

Overview

High resolution allele typing is based on sequencing the positive SSP-PCR reactions. This provides sequence for exon 2 in both the forward and reverse orientations.

The following section describes the steps involved in preparing forward and reverse sequencing reactions from a single SSP-PCR amplicon. There is no requirement for PCR purification in this procedure; the amplicon is simply diluted 1:3 in DNA Diluent buffer or distilled water.

Depending on the number of positive reactions in the low resolution step, the total number of Forward and Reverse sequencing reactions will be between two and eight. The sequencing is performed using Ready Reaction mixes that include the sequencing primer and BigDye™ Terminator Mix.

Performing Sequencing Reactions

Note Use MicroAmp Reaction Tubes or a MicroAmp Optical 96-Well Reaction Plate.

Note Perform thermal cycling using the correct method. Set the sample volume to 10 µL.

To perform the sequencing reactions:

Step	Action
1	Dilute PCR product 1:3 in DNA Diluent. For sufficient sample to sequence in both directions, add 2 µL of PCR product to 6 µL of DNA Dilution Buffer.
2	To separate tubes/plate wells, add 8 µL of each of the Ready Reaction Mixes.
3	To each of the Ready Reaction Mixes in the tubes/plate wells, add 2 µL of diluted PCR product.
4	Seal the tubes with MicroAmp caps or cover the plate with a MicroAmp 2400 or 9600 Full Plate Cover.
5	Centrifuge briefly in a benchtop centrifuge to combine the PCR product and Ready Reaction Mix at the bottom of the tubes/plate wells.
6	Place the tubes/plate in a thermal cycler.

To perform the sequencing reactions: *(continued)*

Step	Action
7	Do 1 cycle of the following: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 seconds
8	Repeat the following for 20 cycles: ♦ 96 °C for 10 seconds ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 10 seconds ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 2 minutes
9	Rapid thermal ramp to 4 °C and hold until ready to purify the sequencing reactions.

Purifying Sequencing Reactions

Following cycle sequencing, the reactions are purified and concentrated to remove unincorporated dye terminators that can appear as artifacts in the sequencing data.

Three methods have been validated for recovering the sequencing ladders following the cycle sequencing reactions. For ease of use as well as increased throughput, we recommend the ethanol precipitation procedure using the 96-well format. For those laboratories that do not have access to a bench-top centrifuge fitted with a 96-well tray adaptor, two alternative methods can be used: a single tube ethanol precipitation method or a Centri-Sep™ spin column procedure.

Precipitating Sequencing Reactions in a MicroAmp 9600 Tray

Perform this step in the same tubes/plate wells as the cycle sequencing reaction. This helps minimize the number of sample transfers and potential sample mix-ups.

Note Use the MicroAmp 9600 Base and Tray Retainer to hold the tubes.

Note If multiple samples are processed in a 96-well format, using a multichannel pipettor can simplify precipitation.

IMPORTANT Perform the steps below without interruption and in the exact order they are given. If the precipitated reactions sit between steps, the signal strength will be decreased.

IMPORTANT Remove the ethanol completely from the precipitated reactions as it may contain unincorporated terminators and dye degradation products.

To perform ethanol precipitation:

Step	Action
1	Prepare a fresh mix: 1.0 mL anhydrous ethanol (EtOH) 40 µL 3M sodium acetate (NaOAc) pH 4.6
2	To each sequencing reaction add 25 µL of the EtOH/NaOAc mix.
3	Replace the strip caps or full plate cover and mix thoroughly by vortexing or inversion.
4	In a centrifuge fitted with a 96-well microtiter tray adaptor, centrifuge for 30 minutes at 1500–3000 *g*. (see “Spin Column Purification” on page 27 to calculate relative centrifugal forces.) **IMPORTANT** Do not refrigerate during the centrifugation run. **IMPORTANT** Be sure that these speeds are within the manufacturer’s recommendations for the centrifuge.
5	Immediately invert the tray on paper towels. Place the inverted tray and paper towel in the centrifuge for 1 minute at 150 *g* to remove the supernatant. **IMPORTANT** This centrifugation step is important to ensure complete removal of dye degradation products.
6	Add 50 µL of 70% ethanol to the tubes. Centrifuge for 5 minutes at 1500–3000 *g*.
7	Immediately repeat step 5 above.

Precipitating Sequencing Reactions in Single Tubes

If there are only a few samples or you do not have access to a centrifuge with a 96-well tray adapter, then a single tube ethanol precipitation procedure may be used.

Step	Action
1	Use a 1.5-mL Microcentrifuge tube for each sequencing reaction.
2	Prepare a fresh mix: 1.0 mL anhydrous ethanol (EtOH) 40 µL 3M sodium acetate (NaOAc) pH 4.6
3	To each 1.5-mL microcentrifuge tube add 25 µL of the EtOH/NaOAc mix.
4	Pipet the entire contents of one extension reaction into one tube of EtOH/NaOAc mix. Mix thoroughly by vortexing.
5	Spin the tubes in a microcentrifuge for 15–30 minutes at maximum speed. **IMPORTANT** Do not refrigerate during the centrifugation run.
6	Carefully aspirate the supernatant with a pipette and discard.
7	Rinse the pellet with 50 µL of 70% ethanol.
8	Spin for 5 minutes in a microcentrifuge at maximum speed. Again, carefully aspirate the supernatant and discard.
9	Dry the pellet in a vacuum centrifuge. Do not over-dry.

Spin Column Purification

We recommend Centri-Sep™ spin columns from Princeton Separations (P/N CS-901). This protocol is different from earlier spin column protocols. The following changes have been made:

- Do not spin the column at speeds >735 *g* (see formula below).
- Do not spin more than 2 minutes.
- The rehydration time has been increased to 2 hours.

Tips for optimizing spin column purification:

- Use one column for each sample. Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed without touching the resin. Make sure that the sample does not touch the sides of the column.
- Spin the column at 325–730 *g* for best results. Use the following formula to calculate the best speed for your centrifuge:

  $g = 11.18 \quad r \quad (\text{rpm}/1000)^2$

  where:

  g = relative centrifugal force

  rpm = revolutions per minute

  r = radius of the rotor in cm
- The entire procedure should be performed without interruption to ensure optimal results. Do not allow the column to dry out.

Rehydrating the Column

Step	Action
1	Gently tap the column to cause the gel material to settle to the bottom of the column.
2	Remove the upper end cap and add 0.8 mL of deionized water.
3	Replace the upper end cap and invert the column a few times to mix the water and gel material.
4	Allow the gel to hydrate at room temperature for ***at least 2 hours***. **Note** Rehydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns that have been stored at 2–6 °C to warm to room temperature before use.

Step	Action
5	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.

Using the Column

Do not allow any interruptions after starting the steps below.

Step	Action
1	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
2	Insert the column into the wash tube provided.
3	Spin the column in a microcentrifuge at 730 *g* for 2 minutes to remove the interstitial fluid.
4	Remove the column from the wash tube and insert it into a sample collection tube (*e.g.*, a 1.5-mL microcentrifuge tube).
5	Remove the extension reaction mixture from its tube and load it carefully on top of the gel material.
6	Spin the column in a microcentrifuge at 730 *g* for 2 minutes. **Note** If using a centrifuge with a fixed-angle rotor, place the column in the same orientation as it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.
7	Discard the column. The sample is in the sample collection tube.
8	Dry the sample in a vacuum centrifuge (no heat) for 10–15 minutes, or until dry. Do not over-dry.

Performing Codon 86 Group-Specific Sequencing

Overview

In some heterozygote analyses, it is possible for multiple pairs of different alleles to generate the same final sequence ladder. For example, the heterozygous sequence from a DRB1*1102/1305 combination is identical to that from a DRB1*11041/1302 allele combination. This situation is generically referred to as an ambiguous heterozygote allele combination. In a majority of these cases, the allele pairs are heterozygous at codon 86. Through the use of codon 86-specific sequencing primers (P/N 4305023), the ambiguity can be resolved.

The heterozygote PCR product generated in the low resolution SSP-PCR is used as a sequencing template in two separate sequencing reactions; one reaction uses a sequencing primer specific for the GTG motif of codon 86 and the other reaction uses a sequencing primer specific for the GGT motif. Homozygous sequences are generated in each reaction, thus separating the two alleles, allowing definitive allele assignment of each individual allele.

Performing Sequencing Reactions

Note Use MicroAmp Reaction Tubes or a MicroAmp Optical 96-Well Reaction Plate.

Note Ensure the thermal cycling is performed with the correct method and the sample volume is set to 10 µL.

To perform the sequencing reactions:

Step	Action
1	Dilute PCR product 1:3 in DNA Diluent. For sufficient sample to sequence with both codon 86 mixes, add 2 µL of PCR product to 6 µL of DNA Dilution Buffer.
2	To separate tubes/plate wells, add 8 µL of each of the codon 86-specific Ready Reaction Mixes.
3	To each of the Ready Reaction Mixes in the tubes/plate wells, add 2 µL of diluted PCR product.
4	Seal the tubes with MicroAmp caps or cover the plate with a MicroAmp 2400 or 9600 Full Plate Cover.
5	Centrifuge briefly in a benchtop centrifuge to combine the PCR product and Ready Reaction Mix at the bottom of the tubes/plate wells.
6	Place the tubes/plate in a thermal cycler.

To perform the sequencing reactions: *(continued)*

Step	Action
7	Do 1 cycle of the following: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 seconds
8	Repeat the following for 20 cycles: ♦ 96 °C for 10 seconds ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 10 seconds ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 2 minutes
9	Rapid thermal ramp to 4 °C and hold until ready to precipitate the sequencing reactions.

To purify the samples, follow the ethanol precipitation protocol described in “Precipitating Sequencing Reactions in a MicroAmp 9600 Tray” on page 25, or “Precipitating Sequencing Reactions in Single Tubes” on page 26 or “Spin Column Purification” on page 27.

Recommendations for ABI PRISM 310 Genetic Analyzer Users

Overview

If you have not previously used the dRhodamine-based sequencing chemistries, the run module and dye set/primer (mobility) file will need to be installed and the instrument (matrix) files made. The run module and dye set/primer (mobility) file are provided on the HLA-DRB BDT Supplemental Diskette. For more information on making the instrument (matrix) files, see Appendix D on page 60.

Note For more information on basic operation and safety, see the ABI PRISM 310 *Genetic Analyzer User's Manual* and the *ABI PRISM 310 Genetic Analyzer Site Preparation and Safety Guide.*

Capillary Preparation

To sequence on the ABI PRISM 310 instrument, use a 47-cm, green dot capillary and Performance Optimized Polymer 6 (POP-6).

Preparing and Loading Samples

IMPORTANT To ensure adequate signal strength, perform the steps below in the exact order they are given.

To prepare and load the samples from a MicroAmp 9600 tray:

Step	Action
1	Resuspend the DNA pellets in 15 µL of the recrystallized formamide provided in the HLA Control and Loading Buffer Module. ! WARNING ! **CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Obtain a copy of the MSDS from Applied Biosystems' Web site or Fax-on-Demand (see "Technical Support" on page 7). Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.** **IMPORTANT** Use only the recrystallized formamide supplied in the HLA Control and Loading Buffer Module. Over time, formamide decomposes to formate. Formate ions are injected preferentially into the capillary, causing a loss of signal intensity.
2	Vortex the tubes, then centrifuge briefly.
3	Denature samples by placing the tray in a heating block or thermal cycler for 2 minutes at 95±5 °C.
4	Vortex the tubes again, then centrifuge briefly.
5	Seal each tube securely using the strip septa.
6	Secure the strip septa with the retainer clips.

To prepare and load the samples from a MicroAmp 9600 tray:

Step	Action
7	Place the sample tray on the autosampler. **IMPORTANT** Do not let the denatured samples sit for more than 48 hours before injection.

To prepare and load the samples in 0.5-mL Genetic Analyzer Sample Tubes:

Step	Action
1	Resuspend the DNA pellets in 15 µL of the recrystallized formamide provided in the HLA Control and Loading Buffer Module. **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Obtain a copy of the MSDS from Applied Biosystems' webpage or Fax-on-Demand (see p. 8). Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.** **IMPORTANT** Use only the recrystallized formamide supplied in the HLA Control and Loading Buffer Module. Over time, formamide decomposes to formate. Formate ions are injected preferentially into the capillary, causing a loss of signal intensity.
2	Vortex the tubes, then centrifuge briefly.
3	Denature each sample by placing in a heating block or thermal cycler for 2 minutes at 95±5 °C.
4	Vortex the tubes again, then centrifuge briefly.
5	Transfer the samples into 0.5-mL Genetic Analyzer Sample Tubes. Label one tube for each sample. **IMPORTANT** Do not transfer to 0.5-mL tubes before the sequences have been heat denatured. This may lead to lower or inadequate signal strengths.
6	Seal each tube with a septum. Press the septa onto the tubes with a slight twist of the thumb.
7	Place the tubes in the 48-well sample tray.
8	Place the sample tray on the autosampler. **IMPORTANT** Do not let the denatured samples sit for more than 48 hours before injection.

Recommendations for Sample Naming

Enter sample information into the sample sheet.

Verify that the Sample Names you enter for the forward and reverse reactions are identical (except for sequence direction), as the MatchTools software searches for sequence pairs with matching Sample Names.

A useful format for Sample Name assignment is the specimen ID, followed by the SSP-PCR group, a left-hand square bracket, and the sequence direction (forward or reverse). For example, a sample that amplified a DRB1 G3/11/6 might have the following file names:

- "Sample ID".G3[F
- "Sample ID".G3[R

IMPORTANT Keep the Sample Name to 26 characters or less.

IMPORTANT If you do not separate the sequence direction from the Sample Name with the left-hand square bracket, the MatchTools software will not correctly group the forward/reverse sequences of each SSP-PCR.

Setting up Default Sample Names

The ABI PRISM 310 Collection Program allows the user to set up default file names of each injection as preferences. Standardized names attached to the sample name can be created and saved.

Step	Action
1	Open the ABI PRISM 310 Collection Software.
2	Under the Window menu, choose Preferences.
3	From Preferences, choose Default File Names.
4	For the Sample File, fill in the three fields as follows: a. Choose <tube number>, in the left pull-down window. b. Click in the middle field and type in <•> (option 8 key stroke). c. Choose <sample name> in the right pull-down window.
5	Click OK.

Sample Sheet Menu choices

The appropriate dye set/primer (mobility) file is "310 HLA-DRB BDT" supplied on the HLA-DRB BDT Supplemental Diskette.

Choose the correct instrument (matrix) file.

Filling in the Injection List

Once the sample sheet is brought in to the Injection List, choose the run module, “310 Run Module HLA-DRB BDT” for every injection. (This module file is supplied on the HLA-DRB BDT Supplemental Diskette. Place it in the Modules folder inside the 310 Collection Software folder.)

When this run module is chosen, the following information is displayed in the injection list to the right of each sample:

Inj. Secs	Inj. kV	Run kV	Run °C	Run Time
30	1.0	15.0	50	28

Signal Strength

For best results, the signal strengths of the analyzed data should range from 100–1000 relative fluorescence units (RFU). Because of normal variations among instruments and other factors, some adjustments may be needed to modify the signal strength.

Increase or decrease the injection time and/or injection voltage appropriately. Injection times greater than 60 seconds are not recommended.

Performing Fluorescent Signal Analysis

Choose which data files to analyze, then analyze and print them using the Sequencing Analysis Software.

Setting the Stop Point in Analysis Parameters

Step	Action
1	Launch the Sequencing Analysis software and add data files to be processed.
2	Open the data file and view the Raw Data.
3	Set the stop point to be at the end of the sequence run. If too many bases are processed, the excess sequence will make it difficult for the MatchTools software to properly define the sequence orientation. Sequence Analysis version 3.0 users may want to set the endpoint in the Basecaller settings to 8 Ns in 10. Be aware that any sequence region containing 8 Ns in 10 bases will be considered an endpoint.
4	Return to the Sample Manager (Sample File Queue) window. Highlight the name of the file just inspected and enter the information for the Stop Point.
5	Enter information for other categories to be customized, such as Basecaller (ABI-CE1), Peak 1 Location (Same as Start Point), Dye Set/Primer file (310 HLA-DRB BDT), and Instrument file.
6	Repeat steps 1–3 for each sequence file.
7	Start analysis.
8	Review the newly analyzed sequences to ensure that the correct analysis parameters were specified. **Note** If you encounter problems with the sequence data generated, refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* or the Troubleshooting Guide in Appendix C on page 46.

Recommendations for ABI PRISM 377 DNA Sequencer Users

Overview

If you have not previously used the dRhodamine-based sequencing chemistries, the run module and dye set/primer (mobility) file will need to be installed and the instrument (matrix) files made. The run module and dye set/primer (mobility) file are provided on the HLA-DRB BDT Supplemental Diskette. For more information on making the instrument (matrix) files, see Appendix D on page 60.

Note For more information on basic operation and safety, see the ABI PRISM 377 *DNA Sequencer User's Manual* and the *ABI PRISM 377 DNA Sequencer Safety Summary*.

Gel Formulation

Prepare a 36-cm well-to-read, 0.2 mm thick, 5% Long Ranger Gel. For details, see the User Bulletin, "Improved Gel Formulations for Extended Sequencing Read Lengths" (P/N 4303614).

! WARNING ! CHEMICAL HAZARD. Long Ranger Gel Solution contains Acrylamide and/or Bis-Acrylamide. Acrylamide and Bis-Acrylamide are poisons, neurotoxins, irritants, carcinogens, and possible teratogens. Acrylamide and Bis-Acrylamide sublime (the solid releases toxic vapor) and are harmful if swallowed, inhaled, or absorbed through the skin. Effects are cumulative. When handling, always wear personal protective equipment (*i.e.*, lab coat, safety glasses, and chemical resistant gloves) and use in a well ventilated area. Thoroughly clean surfaces subject to gel contamination (*i.e.*, binder clips, combs, bench tops, etc.)

Preparing and Loading Samples

IMPORTANT To ensure adequate signal strength, perform the steps below in the exact order they are given.

Preparing the Samples

Step	Action
1	Combine recrystallized formamide and blue dextran/EDTA solution in a 5:1 ratio to prepare the sequencing loading buffer. Make fresh for each use. **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Obtain a copy of the MSDS from Applied Biosystems' Web site or Fax-on-Demand (see p. 8). Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.**

Step	Action
2	Resuspend each DNA pellet in 4µL of sequencing loading buffer.
3	Vortex the tubes, then centrifuge briefly.
4	Denature each sample by placing in a heating block or thermal cycler for 2 minutes at 95±5 °C.
5	Vortex the tubes again, then centrifuge briefly.
6	Place tubes on ice until ready to load.

Recommendations for Sample Naming

Enter sample information into the sample sheet.

Verify that the Sample Names you enter for the forward and reverse reactions are identical (except for sequence direction), as the MatchTools software searches for sequence pairs with matching Sample Names.

A useful format for Sample Name assignment is the specimen ID, followed by the SSP-PCR group, a left-hand square bracket, and the sequence direction (forward or reverse). For example, a sample that amplified a DRB1 G3/11/6 might have the following file names:

- “Sample ID”.G3[F
- “Sample ID”.G3[R

IMPORTANT Keep the Sample Name to 26 characters or less.

IMPORTANT If you do not separate the sequence direction from the Sample Name with the left-hand square bracket, the MatchMaker software will not correctly group the forward/reverse sequences of each SSP-PCR.

Entering default file names for the Sample File

The ABI PRISM 377 Collection program allows the user to set up default file names as preferences. Standardized names attached to the sample name can be created and saved.

Setting up default file names for a Sample File:

Step	Action
1	Open the ABI PRISM 377 Collection software.
2	Under the Window menu, choose Preferences.
3	Hold and release the mouse on Default File Names.

Setting up default file names for a Sample File:

Step	Action
4	For the Sample File, fill in the three fields as follows: a. Choose <lane number> in the left pull-down window. b. Click in the middle field and type in <•> (option 8 key stroke). c. Choose <sample name> in the right pull-down window.
5	Click OK.

Sample Sheet Menu choices

The appropriate dye set/primer (mobility) file is “377 HLA-DRB BDT” supplied on the HLA-DRB BDT Supplemental Diskette.

Choose the correct instrument (matrix) file.

Loading Sample on the ABI PRISM 377

Loading the samples

Step	Action
1	Load the appropriate volume of each sample into a separate lane as previously designated in the sample sheet: ♦ 2 µL for a 24-lane gel ♦ 1.5 µL for a 36-lane gel **IMPORTANT** Load samples into alternate lanes, for example, lanes 1, 3, 5...
2	Prerun samples into the gel for 2 minutes.
3	Pause the electrophoresis prerun.
4	Load the remaining samples into alternate lanes, for example, lanes 2, 4, 6...

ABI PRISM 377 Run Parameters

Prior to collecting data, enter the settings for each of the electrophoresis parameters: Comb, Matrix, Run Module (377 Run Module HLA-DRB BDT), Well-to-Read Distance (36 cm), and Sample Sheet.

The run parameters in the 377 HLA-DRB BDT run module are:

- Voltage: 1.5 kV
- Temperature: 51 °C
- Time: 4 hours
- Filter set E

Note For information about general data collection, see the *ABI PRISM 377 DNA Sequencer User's Manual*.

Performing Fluorescent Signal Analysis

Following Autoanalysis it may be necessary to process the data files. Choose which data files to analyze, then analyze and print them using the Sequencing Analysis software.

Choosing Files for Analysis

Step	Action
1	Launch the Sequencing Analysis software. The Sample Manager window (Sample File Queue in versions 2.1.1 and 2.1.2) appears.
2	Click the Add Files… button. A dialog box appears.
3	Double-click each file to be analyzed, then click Done.
4	Choose the ABI-100 basecaller for each file.

Generating DNA Sequences from Sample Files

Step	Action
1	Set the Start Point and Stop Point using the following procedure. a. Double-click on the first file to be analyzed and view the raw data. b. Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the dialog box. c. Continue scrolling until the cursor is beyond the broad fluorescent peaks at the beginning of the sequencing ladder. d. Set the Start Point to the right of the last broad fluorescent peak. e. Set the Stop Point to be at the end of the sequence run. If too many bases are processed, the excess sequence will make it difficult for the MatchMaker software to define the sequence orientation. Version 3.0 users may want to set the endpoint in the Basecaller settings to 8 Ns in 10. Be aware that any sequence region containing 8 Ns in 10 bases will be considered an endpoint.
2	Return to the Sample Manager (Sample File Queue) window. Highlight the name of the file just inspected and enter the information for the Start Point and Stop Point.
3	Enter information for other categories to be customized, such as Peak 1 Location (Same as Start Point), Dye Set/Primer file (377 HLA-DRB BDT), and Instrument file.
4	Repeat steps 1–3 for each sequence file.
5	Start analysis.
6	Review the newly analyzed sequences to ensure that the correct analysis parameters were specified. **Note** If you encounter problems with the sequence data generated, refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual or* the Troubleshooting Guide in Appendix C on page 46.

Assignment of HLA-DRB Allele Types to Sequence Files

Overview

Each sequence data file is submitted to MatchTools Software for DRB1, DRB3, DRB4, or DRB5 allele determinations. Refer to the *MatchMaker Allele Identification Software User's Manual* for more information.

Using MatchTools Software for Preliminary Analysis

During a preliminary analysis, the MatchTools software performs the following functions:

- Pairs sequences having the same sample name.

  These are typically a forward and a reverse sequence from a single PCR product
- Determines the orientation of each sequence within the pair
- Compares paired sequences with each other and generates a consensus sequence
- Compares the consensus sequence against all of the alleles in the library selected in the Batch Worksheet

MatchTools software then prepares a preliminary Batch Report listing the:

- DRB allele type(s) that best fit(s) each consensus sequence
- Basecalls that differ within each pair of sequences
- Basecalls that display mismatches at constant positions or polymorphic positions for all alleles in the library
- Any insertions or deletions

Note At the 3´ end of the sequences, the reverse direction does not extend as far as the forward sequence. The forward sequence continues to position 270 whereas the reverse sequence is compared against the library only to position 262.

HLA-DRB Allele Libraries

HLA-DRB Allele Libraries to use for each low resolution amplification group/gene:

Allele Groups	Library Name	Codons Included
DR1, DR2, DR3/11/6, DR4, DR8/12	DRB1.L155	14–90
DR7, DR9	DR79.L2	14–78
DR10	DR10.L1	30–88
DRB3, DRB5	DRB35.L17	14–90
DRB4	DRB4.L3	28–88

Using MTNavigator Software for Editing

For manual editing of the sequence data, use MTNavigator Software to:

- Import the Batch Worksheet containing all data files associated with a given sample.
  - The files are typically imported as a Forward/Reverse pair for each SSP allele group or gene sequenced.
  - The sequences are offset such that the nucleotide numbering corresponds to the cDNA sequences as published in the nomenclature reports (*i.e.* the first position is nucleotide #40 in the cDNA sequence). The first 39 bases are either in exon 1 or from the first hypervariable region of exon 2 and therefore not part of the allele library.
- Generate shadow sequences for each pair of sequences to simplify the comparison of basecalling differences between each pair of sequences.
  - Basecall differences between the forward and reverse sequences can be due to a variety of sources, including mobility anomalies or base incorporation anomalies for heterozygote positions (*i.e.,* one base in a heterozygote pair may not be detected at a sufficient level to be identified using the threshold value set in the Batch Worksheet). See Table 3 on page 49 for a list of all currently known reproducible anomalies.

- Perform manual editing of the sequences to resolve basecalling differences between the two orientations.

When the editing is complete, save the changes to the sample files (use the “Save to files...” command in the “Sequences” drop down menu).

Using MatchTools Software for Final Analysis

During a final analysis, use the MatchTools software to:

- Return to the preliminary analysis Batch Worksheet.
- Generate a Final Batch Report by submitting the Batch Worksheet to the MatchTools software. This report includes the:
  - HLA-DRB allele types that best match the edited sequences
  - Basecalls that display mismatches at constant positions or polymorphic positions for all alleles in the library.

Appendix A. HLA-DRB Typing Results Template

Sample Form Use a form like the template below to indicate positive and negative low-resolution grouping results for each sample.

Sample Name	DRB1 G1	DRB1 G2	DRB1 G3/11/6	DRB1 G4	DRB1 G7	DRB1 G8/12	DRB1 G9	DRB1 G10	DRB3	DRB4	DRB5	DRB* ALL

PCR Information	**Attach Photograph of Agarose Gel**
Thermal Cycler:	
Date of Gel:	
Operator:	
Sequencing Cross-reference Information	
Sequencer:	
Date:	
Folder Name:	
Comments:	

Appendix B. HLA-DRB Comparison Table

Low Resolution SSP-PCR Results

The following table presents the normal low resolution SSP-PCR expected results. These relationships are based on the normal haplotype linkage patterns between DRB1 and the other three expressed DRB genes.

DRB1	DRB1	DRB3	DRB4	DRB5
1		–	–	–
2		–	–	+
3/11/6		+	–	–
4		–	+	–
7		–	+	–
8/12 (8)		–	–	–
8/12 (12)		+	–	–
9		–	+	–
10		–	–	–
1	2	–	–	+
1	3/11/6	+	–	–
1	4	–	+	–
1	7	–	+	–
1	8/12 (8)	–	–	–
1	8/12 (12)	+	–	–
1	9	–	+	–
1	10	–	–	–
2	3/11/6	+	–	+
2	4	–	+	+
2	7	–	+	+
2	8/12 (8)	–	–	+
2	8/12 (12)	+	–	+
2	9	–	+	+
2	10	–	–	+

DRB1	DRB1	DRB3	DRB4	DRB5
3/11/6	4	+	+	–
3/11/6	7	+	+	–
3/11/6	8/12 (8)	+	–	–
3/11/6	8/12 (12)	+	–	–
3/11/6	9	+	+	–
3/11/6	10	+	–	–
4	7	–	+	–
4	8/12 (8)	–	+	–
4	8/12 (12)	+	+	–
4	9	–	+	–
4	10	–	+	–
7	8/12 (8)	–	+	–
7	8/12 (12)	+	+	–
7	9	–	+	–
7	10	–	+	–
8/12 (8)	8/12 (12)	+	–	–
8/12 (8)	9	–	+	–
8/12 (8)	10	–	–	–
8/12 (12)	9	+	+	–
8/12 (12)	10	–	–	–
9	10	–	+	–

Appendix C. Troubleshooting Guide

Overview

The protocol and the HLA-DRB BDT Sequencing-Based Typing Kit will provide data that allow high resolution allele assignments. However, certain problematic situations may be encountered as detailed in the tables below. Included in each table are descriptions of the problem, possible causes, and suggestions for resolving the problem.

PCR

Symptom	Possible Cause	Action
No PCR product or weak product	Inappropriate cycling parameters	Check the protocol and confirm the correct method was used.
	DNA not quantitated correctly	Re-quantitate DNA and adjust to 10 ng/µL.
	Degraded DNA	Evaluate on agarose gel and re-extract the DNA if necessary.
	PCR inhibitors in the genomic DNA	Re-extract genomic DNA using one of the recommended methods (see “Blood Collection and DNA Sample Preparation” on page 14).

Sequencing

Symptom	Possible Cause	Action
Weak signal strengths	Inefficient precipitation of sequence ladder	Re-sequence and precipitate, referring to details in “Purifying Sequencing Reactions” on page 24.
	Sequence ladder not denatured properly	Re-sequence, precipitate, and denature, referring to details in “Preparing and Loading Samples” on page 31 for the ABI PRISM 310 DNA Sequencer or “Preparing and Loading Samples” on page 36 for the ABI PRISM 377 DNA Sequencer.
	Source of ethanol	Check reagent quality. Confirm that the ethanol is absolute or anhydrous.

Noisy baseline	Weak signal (less than 50 fluorescent units)	See “Weak signal strengths” details above.
	Signal too strong **Note** If the signal is too strong, multicomponent analysis will not be performed properly.	Dilute the sequence ladder and re-load. For the ABI PRISM 310 user, decrease injection time.
	Incorrect run module/filter set	Repeat the run using the correct module (Run Module HLA-DRB BDT).
	Poor matrix	Make a new matrix. See Appendix D on page 60.
Broad fluorescent terminator artifacts (dye blobs)	Failure to perform 70% ethanol wash	Re-sequence and precipitate, referring to details in “Purifying Sequencing Reactions” on page 24.
	Failure to completely remove supernatant following anhydrous ethanol precipitation	Re-sequence and precipitate, referring to details in “Precipitating Sequencing Reactions in a MicroAmp 9600 Tray” on page 25 or “Precipitating Sequencing Reactions in Single Tubes” on page 26.

Note For more general sequencing problems (*e.g.,* poor resolution, base spacing, etc.), refer to the *ABI PRISM 310 Genetic Analyzer User’s Manual* or the *ABI PRISM 377 DNA Sequencer User’s Manual.*

MatchTools Software

Symptom	Possible Cause	Action
Samples not grouped	Sample names do not match	Use the “Group these files” command in the MatchTools Worksheet menu.
Reverse sequence not automatically reverse complemented by MatchTools software	Too much sequence beyond end of PCR product	Re-analyze with Sequence Analysis software; process only to the end of the sequence.
Heterozygotes not identified by MatchTools software	Base incorporation anomaly	See known anomalies in Table 3 on page 49. Manually edit in MTNavigator software and re-submit.
Allele type not assigned to good quality data	Incorrect allele library selected	Refer to “HLA-DRB Allele Libraries” on page 42.

Anomalies Table Based upon sequences generated with this kit, reproducible anomalies have been observed at some base positions. The table below summarizes and gives examples of those anomalies that have been encountered and may be useful for interpretation/editing of the sequencing data. The majority of anomalies occur in only one sequencing orientation for each base position and can be resolved by reviewing data from the other orientation.

Table 3. Anomalies

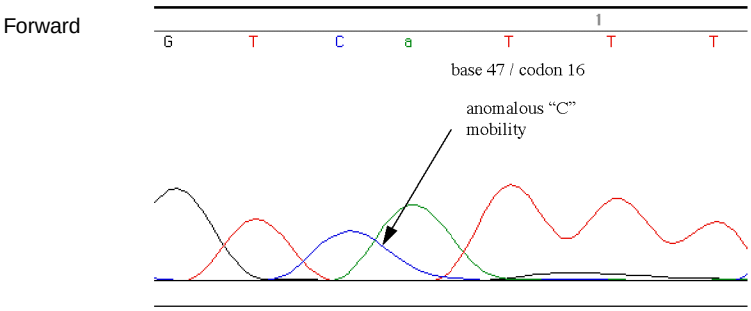
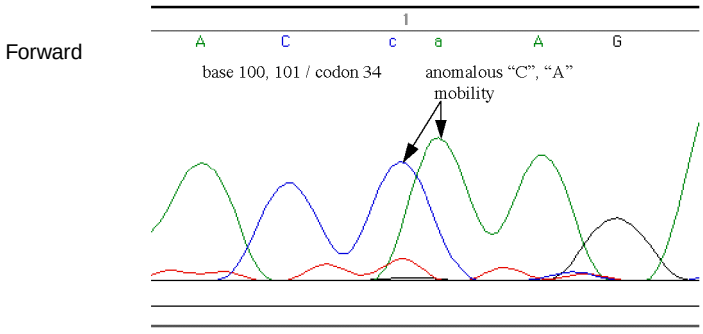
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
47	16	Forward	All (constant position)		CA	CM	mobility shift
							
100, 101	34	Forward	All (constant position)		CA	MM	mobility shift
							

Table 3. Anomalies *(continued)*

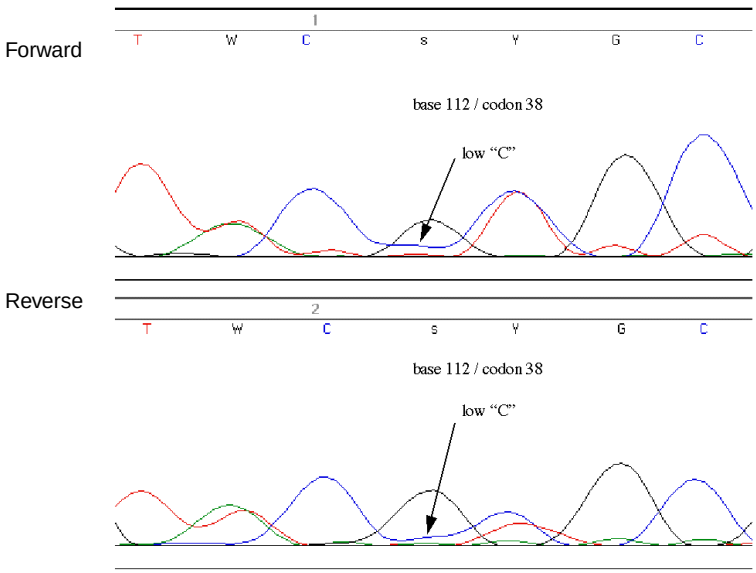
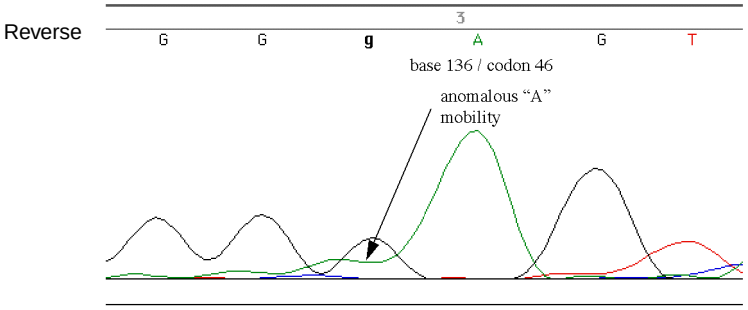
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
112, 113	38	Forward	1301	1401	SY	GY	low C incorporation
112, 113	38	Reverse Complement	1301	1401	SY	GY	low C incorporation
							
136	46	Reverse Complement	All (constant position)		G	R	extra A (shoulder from following A)
							

Table 3. Anomalies *(continued)*

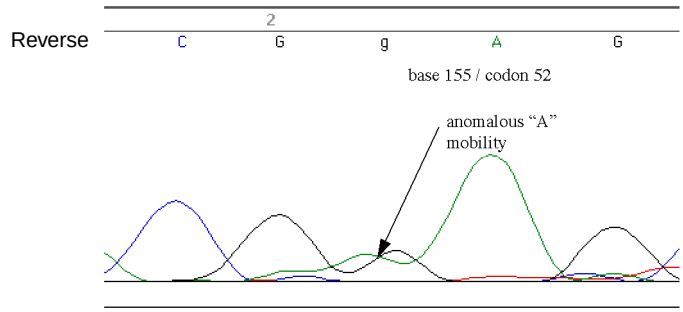
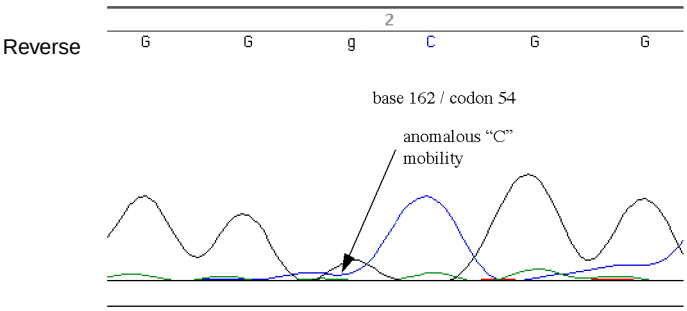
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
155	52	Reverse Complement	All (constant position)		G	R	extra A (shoulder from following A)
							
162	54	Reverse Complement	All (constant position)		G	S	low G with high C background
							

Table 3. Anomalies *(continued)*

Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
169, 170	57	Forward	1302	1303	RA	RR	low G incorporation at position 170
169, 170	57	Reverse Complement	1302	1303	AR	RR	low G incorporation at position 169

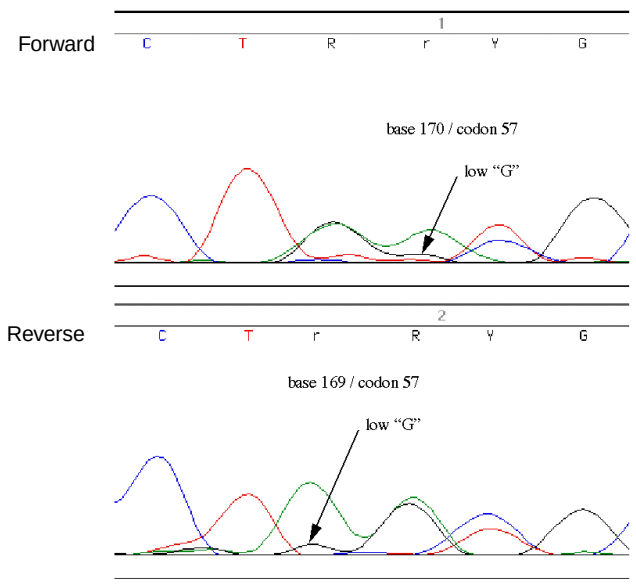


Table 3. Anomalies *(continued)*

Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
170, 171	57	Forward	DRB3* 0101	DRB3* 0202	WY	WT	low C at position 171
170, 171	57	Reverse Complement	DRB3* 0101	DRB3* 0202	WY	AY	low C incorporation at position 170

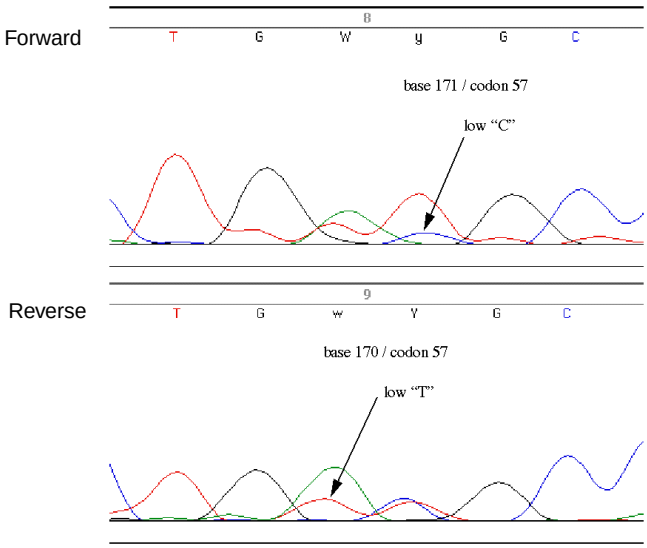


Table 3. Anomalies *(continued)*

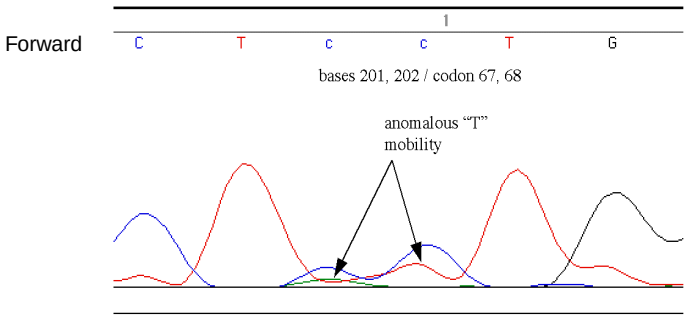
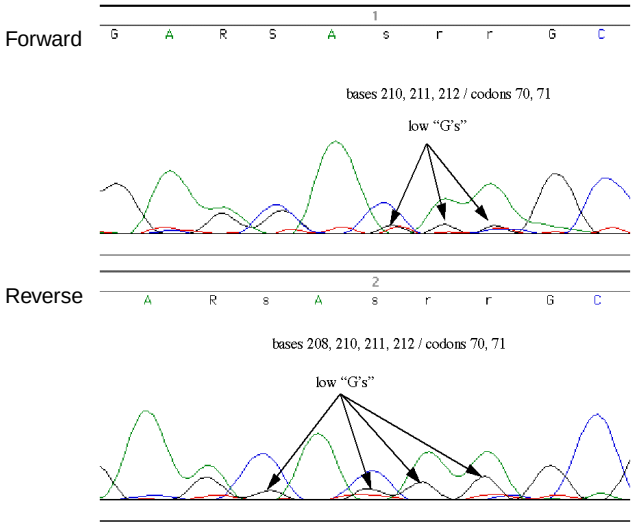
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
201	67	Forward	All (constant position)		C	Y	extra T (shoulder from following T)
							
207-212	69-71	Forward	1301	1401	RSA SRR	RSA CAA	low G at positions 210, 211, 212
207-212	69-71	Reverse Complement	1301	1401	RSA SRR	RCA CAA	low G at positions 208, 210, 211, 212
							

Table 3. Anomalies *(continued)*

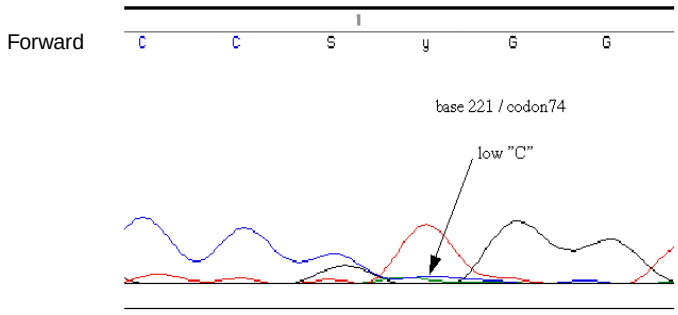
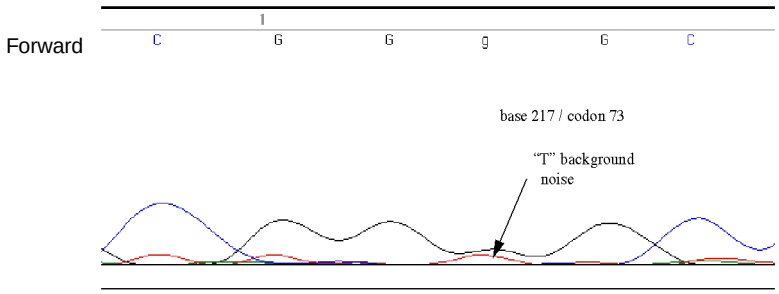
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
212	71	Forward	1301	1303	R	A	low G at position 212
							
217	73	Forward	0301, or DRB3 alleles		G	K	low G with T background noise
							

Table 3. Anomalies *(continued)*

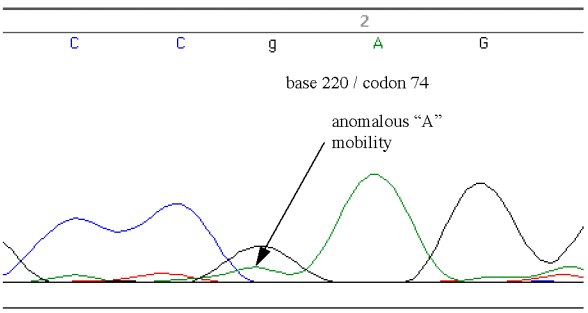
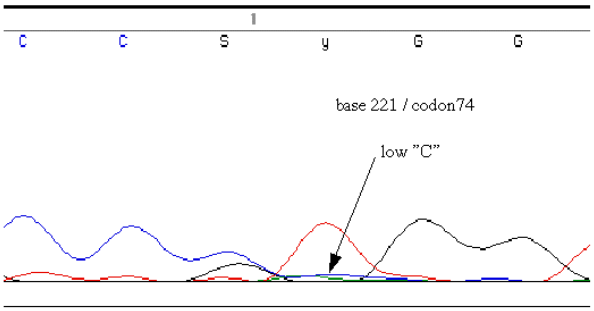
Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
220	74	Reverse Complement	0403		G	R	extra A (shoulder from following A)
Reverse 							
221	74	Forward (377) Reverse Complement (310)	0801	1201	Y	T	low C incorporation
Forward 							

Table 3. Anomalies *(continued)*

Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
256	86	Reverse Complement	codon 86 hetero-zygotes		T	K	anomalous T mobility (see figure for codon 86 below)
257, 258	86	Reverse Complement	codon 86 hetero-zygotes		KK	KT	incomplete resolution of G peaks

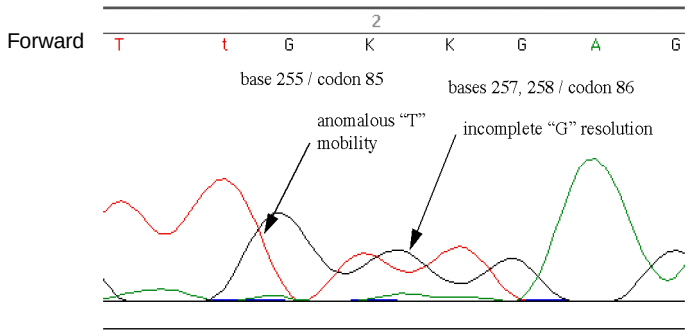
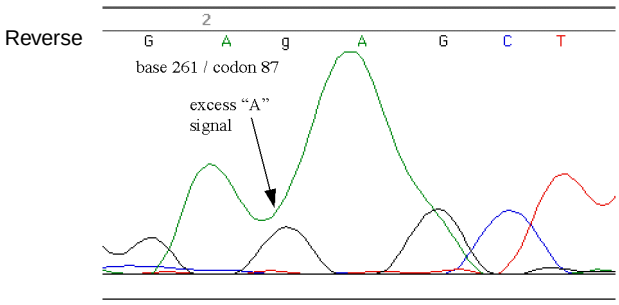
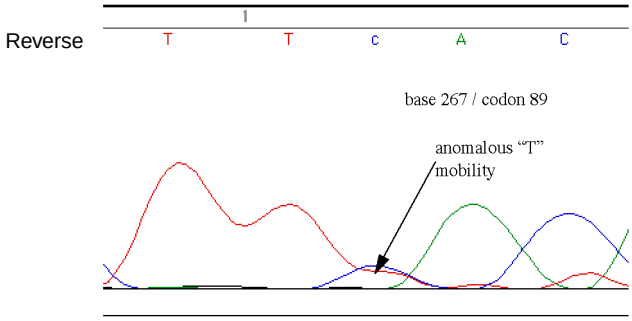


Table 3. Anomalies *(continued)*

Position	Codon	Sequence Orientation[a]	Heterozygote Combination[b]		Correct Base Call	Initial Base Call	Nature of Anomaly
			1st Allele	2nd Allele			
261	87	Reverse Complement	All (constant position)		G	R	excess A signal
							
267	89	Forward	All (constant position)		C	Y	extra T (shoulder from previous T)
							

a. In this table, Reverse Complement refers to the reverse sequences after having been processed by MatchTools. The bases described are those that will be observed in MTNavigator during the editing process.

b. Due to the large number of possible heterozygote allele combinations, the alleles listed in this table are for reference only. For any given sample, it may be helpful to review the published allele sequences to determine whether the same motif combinations are present in the sequences being reviewed.

IUB Codes The code:

A = Adenosine	R = A or G (puRine)
C = Cytidine	Y = C or T (pYrimidine)
G = Guanosine	K = G or T (Keto)
T = Thymidine	M = A or C (aMino)
B = C,G or T	S = G or C (Strong-3H bond)
D = A,G or T	W = A or T (Weak-2H bond)
H = A,C or T	N = aNy base
V = A,C or G	

Appendix D. Instrument (Matrix) Files

Multicomponent Analysis

Multicomponent analysis is the process that separates the four different fluorescent dye colors into distinct spectral components. The four dyes used in the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit are dR110, dR6G, dTAMRA, and dROX.

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM 310 and 377 instruments, the collection software collects light intensities from four specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a “virtual” filter, since no physical filtering hardware (like band-pass glass filters) is used. The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate run module.

In the dRhodamine dyes, dR110 emits at the shortest wavelength and is detected as blue, followed by dR6G (green), dTAMRA (yellow) and dROX (red). Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra among the four dyes. The goal of multicomponent analysis is to isolate the signal from each dye so that there is as little noise in the data as possible.

The precise spectral overlap between the four dyes is measured by running DNA fragments labeled with each of the dyes in separate lanes of a gel or in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples.

The Data Utility software then analyzes the data from each of these four samples and creates an instrument (matrix) file. The instrument file contains tables of numbers with four columns and four rows. These numbers are normalized fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the four dyes.

Multicomponent analysis of sequencing data is performed automatically by the Sequencing Analysis software, which applies a mathematical matrix calculation (using the values in the instrument file) to all sample data.

Making a Matrix from a Sample File

An instrument file can be made from matrix standards or it can be made from a sample file. Using a sample file requires fewer steps than running matrix standards, however the matrix made from a sample file may be different from one made from matrix standards. The quality of a matrix file made from a sample file depends on the quality of the sample file used.

This section describes making a matrix using a BigDye Terminator sequencing standard or a BigDye Terminator sequencing data file. Such an instrument file, containing all three matrices, can be used with either dRhodamine or BigDye chemistries.

For more information on creating a matrix using matrix standards, refer to the *ABI PRISM dRhodamine Matrix Standards User Bulletin* (P/N 904917).

If problems arise, such as spectral overlap in the analyzed data, a new matrix can be made from any terminator sequencing sample by following the instructions below.

Run the Sequencing Standard

Make one sample of the BigDye Terminator Cycle Sequencing Standard (P/N 4304154) following the instructions on the product insert. If using a sequencing data file, follow the steps below substituting the appropriate file name.

Make Copies of the Standard Run

Make three duplicate copies of the BigDye terminator standard run before creating a matrix:

Step	Action
1	Open the Run folder, and highlight the sample file by clicking on the name.
2	Open the File menu and choose Duplicate. A duplicate is created, called “Sample file copy”.
3	Open the File menu and choose Duplicate two more times to create “Sample file copy-2” and “Sample file copy-3.”

View Matrix Run Raw Data

Use the Data Utility software to view raw data of the matrix runs:

Step	Action
1	Launch the Data Utility software.
2	Under File, choose Open.
3	Search for the Run Folder containing the four sample files (one original and 3 duplicates).
4	Click on the first sample. The corresponding open windows are: ♦ Raw data ♦ Analyzed ♦ EPT ♦ Information
5	Verify that the baseline is stable and peak heights are above 100 fluorescent units in the raw data.
6	Choose a scan point (*e.g.* 2000) that occurs after the first peaks, though still in the beginning of the run.

Generate the Terminator Matrix

The matrix standards are analyzed mathematically to create matrices.

To generate a matrix :

Step	Action
1	From the Utilities menu, select Make Matrix... The Make Matrix dialog box appears.
2	Designate the files for C, A, G, and T data. Example: a. Click the C button, then choose “Sample file” in the Run Folder. b. Do the same for the A (“Sample file copy”), G (Sample file copy-2”), and T (“Sample file copy-3”) buttons. c. Use the default settings of 2000 for Start at and 1500 for Points.
3	Click New File, name the matrix appropriately (*e.g.*, HLA BDT), and save it to the ABI Folder in the System folder.
4	Click T7 Terminator Matrix.
5	Click OK. The message “Make matrix successfully completed” appears.

To generate a matrix *(continued)*:

Step	Action
6	If an error message occurs, modify the default values in the Make Matrix dialog box. Make a new matrix.

Note If you get an error message, and the software will not make a matrix, you may have designated the wrong files. It is possible, though rare, that the signal is too weak to make a matrix. If the signal is inadequate to make a matrix, rerun the standard or use another dRhodamine/BigDye chemistry sequencing run.

Complete the Other Matrices

Repeat steps 1–5 on page 62 for making the Dye Primer Matrix and the Taq Terminator Matrix of the instrument file.These two other matrices are needed by the sequencing analysis software to analyze a BigDye Terminator sequencing sample.

Be sure to select Update File instead of New File when repeating step 3. The end result is a complete instrument file that can be used for analyzing Big Dye and dRhodamine chemistries.

Evaluate Matrix Quality

To evaluate the quality of the matrix:

Step	Action
1	Launch the Sequencing Analysis software.
2	In the Sample Manager, add the sample file used to make the matrix.
3	Select the new matrix under Instrument File.
4	Reanalyze the data file.
5	Look at the Analyzed data window. Peaks of only one color should be seen (Figure 1). Peaks under peaks indicate a poor or incorrect matrix (Figure 2).
6	If the sequence quality is poor, do one of the following: a. Modify the Make Matrix default values. b. See Note on preceding page.

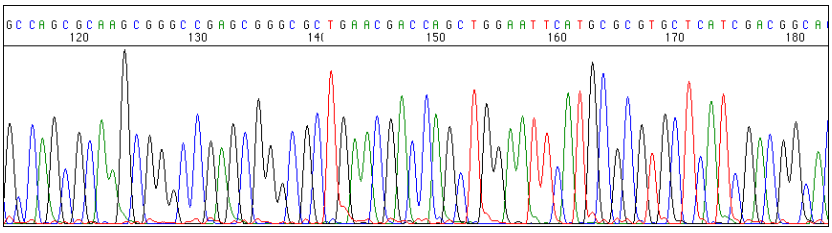


Figure 1 Correct matrix

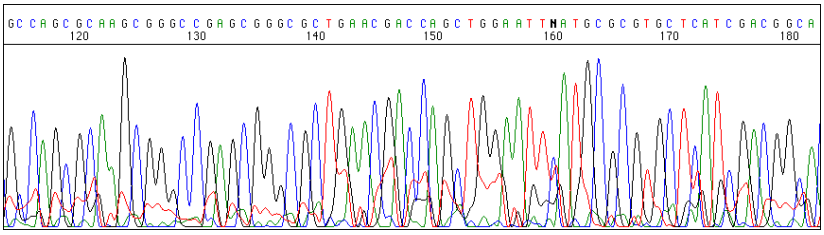


Figure 2 Incorrect matrix

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